

Effect of Cytosine Arabinoside and 5-Iodo-2'-Deoxyuridine on a Cytomegalovirus Infection in Newborn Mice¹

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Received for publication 22 September 1975

Murine cytomegalovirus was inhibited by 0.6 to 1.2 μg of cytosine arabinoside per ml and by 0.3 to 0.6 μg of 5-iodo-2'-deoxyuridine in mouse embryo fibroblast cells. Human cytomegalovirus was inhibited by similar concentrations of the two drugs in WI-38 cells. Intraperitoneal inoculation of suckling mice with 10^{4-5} plaque-forming units of murine cytomegalovirus provides a model for disseminated human cytomegalovirus infection in human newborn infants and is characterized by a widespread infection involving the liver, spleen, lung, kidney, and brain with a 70 to 90% mortality in 7 to 9 days. Treatment with 12.5 mg of cytosine arabinoside per kg or 25 mg of 5-iodo-2'-deoxyuridine per kg twice daily for 8 days had no effect on final mortality or the pathogenesis of the infection with the exception of reduced viral titers in the spleen of 5-iodo-2'-deoxyuridine-treated animals. These data indicate that neither cytosine arabinoside nor 5-iodo-2'-deoxyuridine are effective in the treatment of murine cytomegalovirus infections in suckling mice and suggest that they may be of limited value in the treatment of severe cytomegalovirus infections in human neonates.

Cytomegalovirus infection of humans (HCMV) is manifested by a variety of disease patterns including: (i) an intrauterine infection involving multiple organ systems (6); (ii) a chronic infection affecting cells of the peripheral blood, kidney, liver, or lung in patients with depressed host resistance associated with some underlying disease process or immunosuppressive therapy (5, 10); (iii) a mononucleosis-like syndrome in patients perfused with large amounts of fresh, whole blood (14); and (iv) an asymptomatic infection of either children or adults (25). From 1 to 3% of all infants born in the United States have evidence of HCMV infection during the neonatal period (7, 24). Although most of these infections are asymptomatic, approximately 10% of these infants will exhibit signs of visceral involvement during the neonatal period, and a significant number of these will subsequently develop abnormalities of the central nervous system (16). Conservative estimates based on the present national birth rate indicate that approximately 3,000 to 6,000 infants born in the United States annually will suffer some degree of neurological damage as a result of congenital HCMV infection. Because of the high incidence of neu-

rological sequelae after symptomatic congenital infection, several workers have attempted to treat these infections with either cytosine arabinoside (ara-C) (3, 13, 15, 21) or 5-iodo-2'-deoxyuridine (IUdR) (1), both of which have been shown to inhibit the replication of HCMV in vitro (22, 23). Although treatment has resulted in decreased urinary excretion of virus (13, 15), clinical improvement has been difficult to evaluate, and there has been no definitive evidence that the development of neurological residua was altered. Furthermore, information regarding success or failure of therapy has been obtained from individually treated cases. Results of controlled studies in humans are not yet available.

Recognizing the need to evaluate the effectiveness of chemotherapeutic agents for HCMV infections, we have developed an animal model utilizing suckling mice infected with the murine strain of cytomegalovirus (MCMV), which is similar to the generalized infection of human newborn infants. To determine the potential efficacy of ara-C and IUdR in the treatment of cytomegalovirus infections, the following studies were carried out: (i) a comparison of the susceptibility of HCMV and MCMV to ara-C and IUdR in vitro, (ii) a delineation of the effect of treatment with ara-C or IUdR on mortality of infected mice, (iii) a determination of the patho-

¹ Publication no. 21 from the Cooperative Antiviral Testing Group of the National Institute of Allergy and Infectious Disease, Bethesda, Md.

genesis of the infection in suckling mice, and (iv) an evaluation of the effect of drug therapy on the pathogenesis of the infection.

MATERIALS AND METHODS

Animals. Suckling mice (5 to 7 days of age) from Swiss-Webster pregnant females (Simonsen Laboratories, Inc., Gilroy, Calif.) were randomized and segregated into groups of 10 per lactating female.

Media. Eagle minimum essential medium (MEM) or Eagle basal medium consisted of Earle balanced salt solution supplemented with vitamins and amino acids (Grand Island Biological Co., Grand Island, N.Y.), glutamine (300 $\mu\text{g}/\text{ml}$), penicillin (100 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), NaHCO_3 (2 mg/ml), and 10% fetal calf serum (Grand Island Biological Co.). Where noted, amphotericin B was added at a final concentration of 2.5 $\mu\text{g}/\text{ml}$.

Cell cultures. The preparation of primary mouse embryo fibroblast (MEF) cells has been described previously (11). Human embryonic lung (WI-38) cells obtained from Leonard Hayflick, Stanford University, Palo Alto, Calif., were propagated in Eagle basal medium in 32-ounce (0.473-liter) prescription bottles and used between passages 20 and 30.

Viruses. The Smith strain of MCMV was obtained from June Osborn, University of Wisconsin, Madison, Wis. Virus pools were prepared by inoculating weanling mice intraperitoneally (i.p.) with 10^8 plaque-forming units (PFU) of MCMV and removing the salivary glands 14 days later. The glands from a large number of mice were pooled and prepared as 10% homogenates (wt/vol) in MEM. Virus pools prepared in this manner regularly titered 10^8 PFU per ml when assayed on secondary MEF cells. The HCMV strains used were the AD-169 strain from the American Type Culture Collection (Rockville, Md.), the *DrLu* strain isolated from the lung biopsy of an immunosuppressed patient with HCMV pneumonitis, and the *DiUr* strain isolated from the urine of an infant with congenital HCMV infection. These isolates were passaged four times in confluent monolayers of WI-38 cells. On these cells the AD-169 pool titered 10^6 PFU/ml, and the *DrLu* and *DiUr* pools titered 10^4 PFU/ml.

Virus assays. Both the MCMV and HCMV strains were assayed by employing a modification of a technique originally described by Wentworth and French that utilizes sequential agarose overlays (27). To assay MCMV, secondary MEF monolayers were prepared from primary cells by seeding plastic petri dishes (10 by 30 mm; Falcon Plastics, Oxnard, Calif.) with 10^6 MEF cells in 2 ml of MEM. The cells were incubated for 48 h at 37 C in 5% CO_2 with humidity, the growth medium was removed, the monolayers were washed once with 1 ml of phosphate-buffered saline (PBS), and each plate was inoculated with 0.2 ml of a suitable dilution of virus. Virus was allowed to adsorb for 1 h at 37 C, and the plates were overlaid with 2 ml of MEM containing 0.3% (wt/vol) agarose (Van Waters and Rogers, San Francisco, Calif.). These plates were incubated at 37 C in 5% CO_2 with humidity, and a second overlay of 1 ml was

added on day 2. Five days after infection, a third 1-ml overlay consisting of 0.75% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.), MEM, and neutral red (Matheson, Coleman, and Bell, Cincinnati, Ohio) at a final concentration of 1:40,000 was added. After overnight incubation, MCMV microplaques were enumerated with the aid of a stereoscopic microscope. The procedure for assaying HCMV was similar. Confluent monolayers of WI-38 cells were inoculated with virus and overlaid as described above. Sequential 1-ml overlays were added on days 3, 6, and 9 after initial infection. On day 12 the plates were overlaid with agar-neutral red medium, and after overnight incubation plaques were counted under the stereoscopic microscope.

Preparation of murine tissues for viral assay. Liver, lung, spleen, kidney, and brain tissues were excised from 10 suckling mice at various times after infection, pooled by organ group, and weighed. A volume of MEM calculated to result in a 10% (wt/vol) suspension was added, and the tissues were homogenized in a glass tissue grinder (Kontes Glass Co., Vineland, N.J.). The homogenates were centrifuged, and the supernatants were collected and frozen at -70 C until assayed for MCMV. Blood was obtained from individual animals by cardiac puncture, pooled, and homogenized without dilution in glass tissue grinders. Urine was collected during exsanguination into glass test tubes and diluted 1:2 or 1:5 in MEM with amphotericin B. If necessary the pH of the urine samples was adjusted to neutrality with 1 N NaOH.

Antiviral drugs. ara-C (Cytosar, Cytarabine) was kindly supplied by the Upjohn Co. (Kalamazoo, Mich.). The lyophilized powder was diluted to the desired concentration in PBS and administered in 0.05-ml volumes to suckling mice by i.p. injection. IUdR was obtained from Calbiochem (La Jolla, Calif.). The drug was dissolved in PBS adjusted to pH 11 with 1 N NaOH and then readjusted to pH 9 with 1 N HCl, and the appropriate concentration of drug was administered in 0.05-ml volumes to suckling mice by i.p. injection.

Assays of antiviral activity in vitro. The sensitivity of MCMV and HCMV strains to ara-C and IUdR was determined by incorporating serial twofold dilutions of each drug into each sequential overlay as described previously (11). The inhibitory level was defined as that concentration of drug which resulted in a 50% reduction of the number of plaques in the untreated control. Dilutions of virus were utilized that resulted in 30 to 50 PFU per plate. The pH of the media remained between 7.0 and 7.4 throughout the course of the assay.

RESULTS

Sensitivity of MCMV and HCMV strains to ara-C and IUdR in vitro. To determine whether efficacy against MCMV infections could be applicable to HCMV disease, the susceptibilities of both MCMV and HCMV to ara-C and IUdR in vitro were compared. Fifty percent

plaque inhibition levels were determined for MCMV in secondary MEF cells and for HCMV (AD-169, *DrLu*, and *DiUr*) in WI-38 cells. The results of two experiments are presented in Table 1. The data indicate that both MCMV and HCMV are susceptible to both ara-C and IUdR. There does not appear to be any appreciable difference in the level of susceptibility of either the human or the murine virus to either compound. Concentrations of ara-C greater than 12.5 $\mu\text{g/ml}$ on MEF cells and 5 $\mu\text{g/ml}$ on WI-38 cells were toxic to control cultures. Concentrations of IUdR of 100 $\mu\text{g/ml}$ on MEF cells and 10 $\mu\text{g/ml}$ on WI-38 cells were not toxic to control cultures and inhibited plaque formation completely.

Treatment with ara-C or IUdR. To evaluate the effect of ara-C and IUdR on the mortality of mice infected with MCMV, 5- to 7-day-old suck-

ling mice were infected by the i.p. route with $10^{4.5}$ PFU, an inoculum of virus calculated to result in 80 to 95% mortality. Treatments with 25 and 12.5 mg of ara-C per kg or 50 and 25 mg of IUdR per kg were administered twice daily by i.p. injection for a period of 8 days starting immediately after infection. In preliminary experiments, it had been determined that drug concentrations greater than 25 mg/kg per dose for ara-C or 100 mg/kg per dose for IUdR, employing the above regimen, were lethal for our animals. The therapeutic effect of treatment of groups of 30 animals with ara-C is illustrated in Fig. 1. In this experiment 98% of the animals in the virus control group treated only with drug diluent died by day 9. No adverse effects were seen in groups of uninfected animals treated with drug diluent. Mice infected with MCMV and treated with 25 or 12.5 mg of ara-C per kg had a final mortality of 88 and 86%, respectively. Treatment with these dosages of drug usually resulted in some toxicity, as manifested by the 15 to 25% mortality in the drug control groups. ara-C therapy at these or lower doses of drug had no effect on the outcome of this experimental infection. The effect of IUdR therapy on mortality utilizing twice daily dosages of 50 and 25 mg/kg for 8 days is shown in Fig. 2. In this experiment the cumulative mortality for the virus control group treated with drug diluent was 80% by day 9 after infection. In both treatment groups, 75% of the animals had died by this time. There was no effect of IUdR therapy at these or lower doses of drug on either the time of death or the final mortality. No deaths

TABLE 1. Susceptibility to ara-C and IUdR of MCMV and HCMV strains in MEF and WI-38 cells

Strain	50% inhibitory levels ($\mu\text{g/ml}$)		
	IUdR	ara-C	
MCMV	0.6	0.3	
	1.2	0.6	
HCMV			
	AD-169	0.2	0.5
		0.2	0.5
	<i>DrLu</i>	0.2	0.5
		0.2	0.2
	<i>DiUr</i>	0.2	0.5
	0.2	0.2	

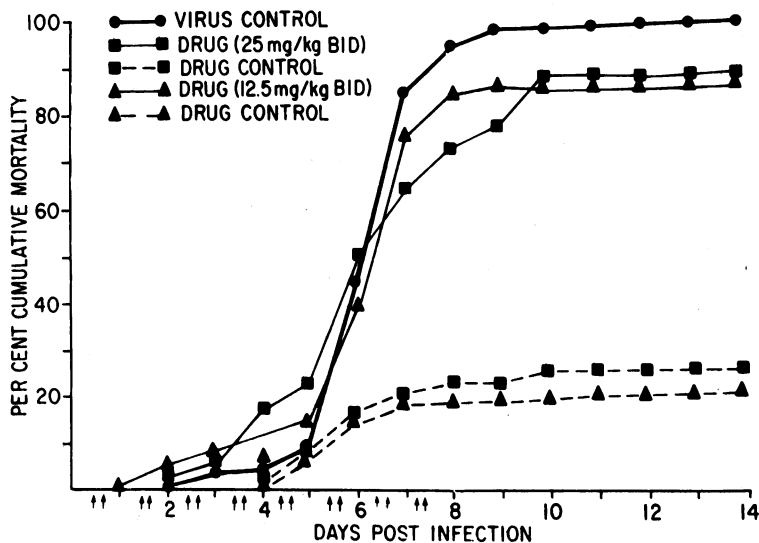


FIG. 1. Effect of ara-C (12.5 mg/kg twice daily for 8 days) on the mortality from a MCMV infection in suckling mice. Arrows indicate times of drug administration.

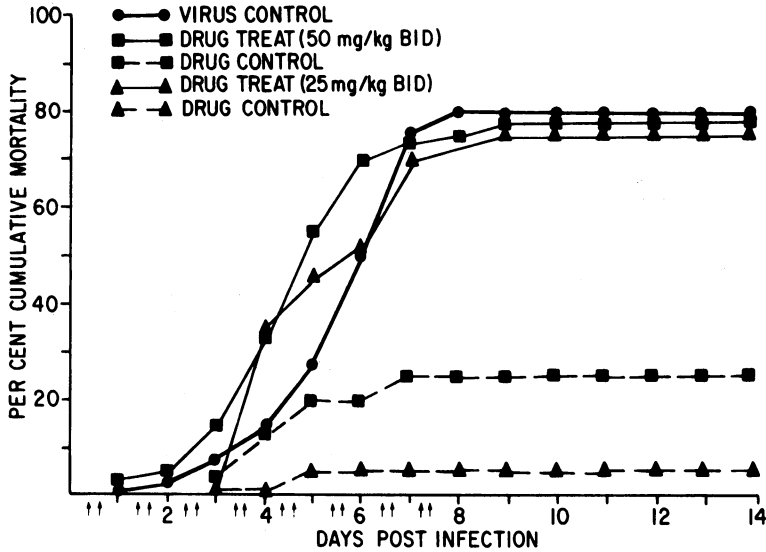


FIG. 2. Effect of IUdR (25 mg/kg twice daily for 8 days) on the mortality from a MCMV infection in suckling mice. Arrows indicate times of drug administration.

occurred among groups of uninfected control animals treated with the drug diluent (PBS at pH 9).

Effect of ara-C and IUdR therapy on the pathogenesis of MCMV infection. To define the effect of ara-C and IUdR on the pathogenesis of this infection, 5- to 7-day-old suckling mice were inoculated i.p. with approximately $10^{4.5}$ PFU of virus. The typical course of infection in control animals inoculated with MCMV and treated twice daily with PBS is illustrated in Fig. 3. Replication of virus in major target organs (liver, spleen, lung, and kidney) was evident within 24 h after infection. Viral titers rose rapidly in these organs to 10^5 to 10^6 PFU/g of tissue by day 4, and remained at this level throughout the course of the disease. A viremia was detectable at 48 h, and seeding of brain tissue with virus occurred at approximately 72 h. To evaluate the effect of therapy with ara-C on the pathogenesis of MCMV infection, mice were treated with 12.5 mg/kg twice daily for 8 days beginning immediately after infection. There was no significant delay or inhibition of viral replication in any of the target organs tested (Fig. 4). In another experiment, treatment with 25 mg of ara-C per kg twice daily for 6 days also failed to alter MCMV pathogenesis. To define the effects of IUdR treatment on pathogenesis of this infection, a similar protocol to that described above was used. In the untreated control mice (Fig. 5) the pathogenesis of the MCMV infection was similar to that described previously. In the animals treated with 25 mg of IUdR per kg twice daily for 8 days (Fig. 6)

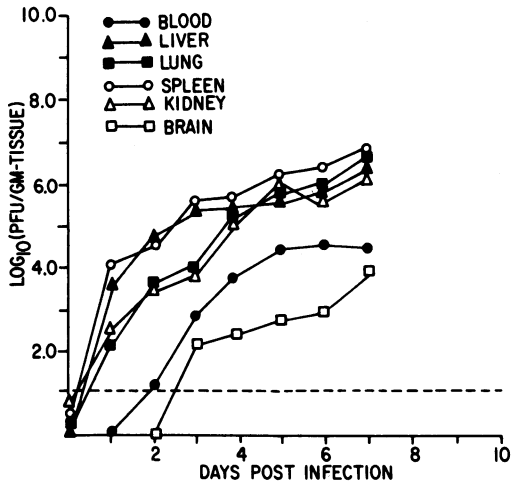


FIG. 3. Effect of ara-C (untreated control) on the pathogenesis of a MCMV infection in suckling mice.

viral titers in the spleen on day 3 were reduced from $10^{6.3}$ PFU/g of tissue in the control animals to $10^{3.8}$ PFU/g of tissue in the treated animals and from 10^7 to $10^{5.7}$ PFU/g of tissue on day 5. No effect on viral replication was observed in any other organ during the course of infection. To further assess the effect of IUdR therapy on the pathogenesis of this infection, pooled 0.1-ml volumes of urine were obtained from groups of treated and untreated animals at daily intervals after infection. The samples were immediately diluted in buffered tissue culture medium, adjusted to neutral pH, and assayed for the presence of virus. The results of

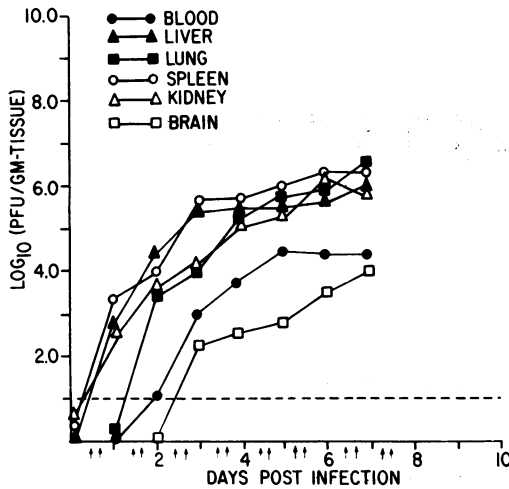


FIG. 4. Effect of ara-C (12.5 mg/kg twice daily for 8 days) on the pathogenesis of a MCMV infection in suckling mice. Arrows indicate times of drug administration.

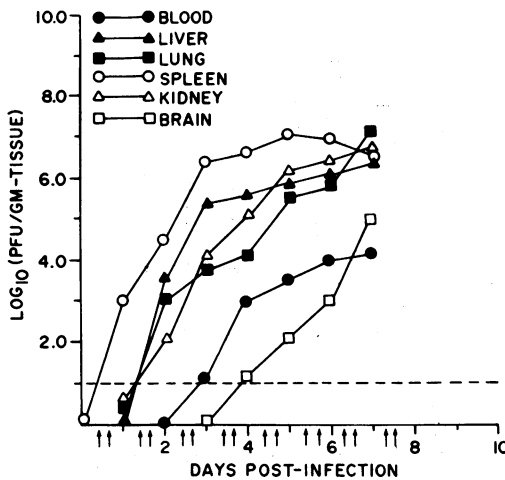


FIG. 5. Effect of IUDr (untreated control) on the pathogenesis of a MCMV infection in suckling mice.

two such experiments are shown in Table 2. Because of the small volumes of the urine samples, titers of virus lower than approximately 10^2 PFU/ml of urine could not be detected. MCMV was not found in the urine of untreated control animals until days 4 and 6, and titers of virus were quite low. Although the data suggest that IUDr treatment may have delayed the appearance of virus in the urine by 1 day, the number of plaques formed at the lowest dilution assayed were so few that we are unable to conclude that drug treatment had any effect on the urinary excretion of virus in suckling mice.

DISCUSSION

To more closely examine the parameters involved in the chemotherapy of cytomegalovirus infections, we developed an animal model of a disseminated MCMV infection that simulates several aspects of the disease process in severely ill human newborn infants. To strengthen the validity of this model system, we determined the susceptibility of both HCMV and MCMV strains to IUDr and ara-C in vitro. The data indicated that replication of both HCMV and MCMV was inhibited by similar concentrations of both ara-C and IUDr. In the suckling mouse infection, treatment with maximally tolerated doses of ara-C or IUDr failed to alter mortality. With the exception of suppression of viral replication in the spleen of IUDr-

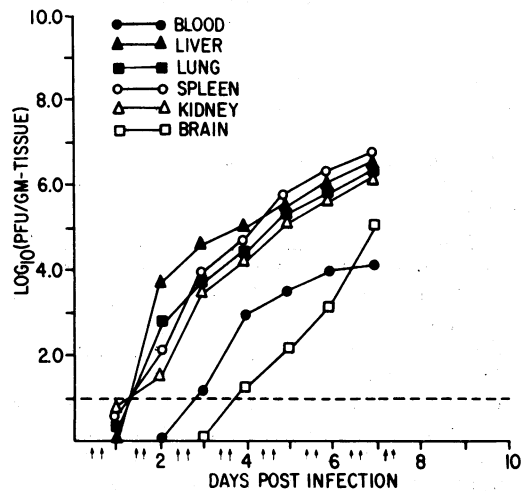


FIG. 6. Effect of IUDr (25 mg/kg twice daily for 8 days) on the pathogenesis of a MCMV infection in suckling mice. Arrows indicate times of drug administration.

TABLE 2. Effect of IUDr^a on urinary excretion of MCMV in suckling mice

Days post-infection	Log ₁₀ PFU of virus/ml of urine			
	Expt 1		Expt 2	
	Control	Treated	Control	Treated
1	— ^b	— ^b	— ^c	—
2	—	—	—	—
3	—	—	—	—
4	—	—	2.4	—
5	—	—	2.7	2.4
6	3.0	—	3.2	2.7

^a Dosage was 25 mg/kg twice daily for 8 days beginning immediately after viral infection.

^b —, <2.2 log₁₀ PFU of virus/ml of urine.

^c —, <1.9 log₁₀ PFU of virus/ml of urine.

treated animals, treatment with either drug did not significantly affect the pathogenesis of the infection. In contrast to the results demonstrated in the treatment of the human disease, we were unable to show any definitive effect of drug therapy on urinary excretion of virus.

Several possible explanations for the failure of ara-C or IUdR therapy to reduce final mortality or to significantly alter the pathogenesis of MCMV infection of suckling mice can be cited. (i) The dosages of the drugs utilized and the duration of therapy may have been insufficient to successfully treat the animals. This seems unlikely in that maximally tolerated dosages of drug were used and continued for a total of 8 days, at which time most of the animals had already died. (ii) Because of immature host resistance mechanisms in newborn mice and the enhanced susceptibility of suckling mice to MCMV infection when compared to adult mice (19), this infection may have been so virulent as to preclude the effectiveness of these antiviral compounds. Treatment of adult animals with less virulent infection, however, would be needed to answer this question. (iii) The antiviral agents themselves may have exerted an immunosuppressive effect and interfered with elimination of the virus by the host. Stevens and co-workers (26) demonstrated in a double-blind controlled study that patients with disseminated herpes zoster and underlying stage III or IV lymphoma who were treated with ara-C had a more prolonged clinical course than placebo-treated patients, presumably because of the immunosuppressive effect of ara-C. (iv) The MCMV infection itself may have resulted in immunosuppression and inhibited the elimination of virus from infected tissues. Several workers have demonstrated the suppression of antibody production (8, 9, 14, 15), skin graft rejection (19), and lymphocyte reactivity (D. K. Kelsey, G. A. Olsen, and J. C. Overall, Jr., *J. Reticuloendothel. Soc.* 16:27a, 1974) during the acute stages of MCMV infection. (v) The virus could have been resistant to the antiviral drugs. However, MCMV appeared to be quite susceptible to both ara-C and IUdR in mouse cells at levels similar to those we have previously reported for *Herpesvirus hominis* type 2 (11, 12). (vi) It is possible that inhibitory concentrations of drug were not achieved in the target organs or cells in which the virus was replicating. In previous reports from this laboratory, both ara-C and IUdR failed to reduce final mortality in disseminated *H. hominis* type 2 infection of suckling mice, primarily because of inadequate concentrations of the antiviral compounds in the central nervous system

and, to a lesser degree, in the lung (11, 12). In this same *H. hominis* type 2 model infection, however, treatment with dosage regimens similar to the one used in the current study either completely inhibited or significantly delayed the onset of viral replication in the liver and spleen, suggesting that effective levels of drug were achieved in those organs. In contrast, little or no alteration of viral replication in any of the target organs tested was observed in the MCMV infection of suckling mice. The reason for this difference is not clear, particularly in view of the similar susceptibility of both *H. hominis* type 2 and MCMV to the two drugs in vitro. It is possible that MCMV replication takes place in different cells in the liver and spleen, and that these cells are not penetrated as well by ara-C and IUdR or that these drugs are less active in MCMV-infected cells. Supporting this hypothesis is the fact that several workers (2, 4, 20) have demonstrated that antiviral compounds have different activity against the same virus in different cell lines, even if the cells are from the same species. It appears, therefore, that the failure of ara-C and IUdR therapy in MCMV infection of suckling mice is due primarily to inadequate concentrations of biologically active drug within the appropriate cells of the critical target organs along with a drug and viral induced immunosuppression of host resistance.

The failure of ara-C and IUdR therapy in MCMV infection of suckling mice does not preclude their possible beneficial effect in the treatment of HCMV disease. Symptomatic congenital HCMV infection in human newborn infants is a much less virulent infection that rarely results in death in the neonatal period. Although it must be recognized that results from antiviral chemotherapy experiments in animals cannot be directly extrapolated to the human situation, our results suggest that neither ara-C nor IUdR may be of value in the treatment of HCMV. Further animal studies as well as controlled clinical trials in humans will be required before this issue can be resolved.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Contract no. 70-2133 from the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases and by Public Health Service grant no. AI 10217 from the National Institute of Allergy and Infectious Diseases. J. C. O. is an investigator of the Howard Hughes Medical Institute.

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